

# Isolation and molecular identification of *Ascomycetes* in sediments and waters of the Gulf of Aqaba, Red Sea

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## ABSTRACT

Using molecular approach, we aimed to identify fungal phylotypes that exist in the Gulf of Aqaba, Red sea. Several samples were taken from sediments and seawater of three locations along 26 kilometers at 5 m depth. 18S small subunit rRNA gene was targeted for PCR amplification and sequencing. Partial sequences introduced as query in BLASTN phylogenetic analysis revealed 100% identity with *Ascomycota*, namely, *Aspergillus* sp. *Penicillium* sp. and its closely related *Eupenicillium* sp. The top scorer species in this analysis were *Aspergillus sydowii*, *Aspergillus wentii*, *Aspergillus flocculosus*, *Penicillium expansum* and *Eupenicillium javanicum* with 98% - 100% identity. Phylogenetic analyses demonstrates close relatedness among isolated fungi and potential association with *Ascomycetes*. This study reports a new geographical location in which facultative marine *Ascomycetes* exist in, and sheds some light on fungal diversity in Gulf of Aqaba.

**Keywords:** Marine Fungi; *Ascomycete*; 18S ssu rRNA; Gulf of Aqaba; Red Sea

## 1. INTRODUCTION

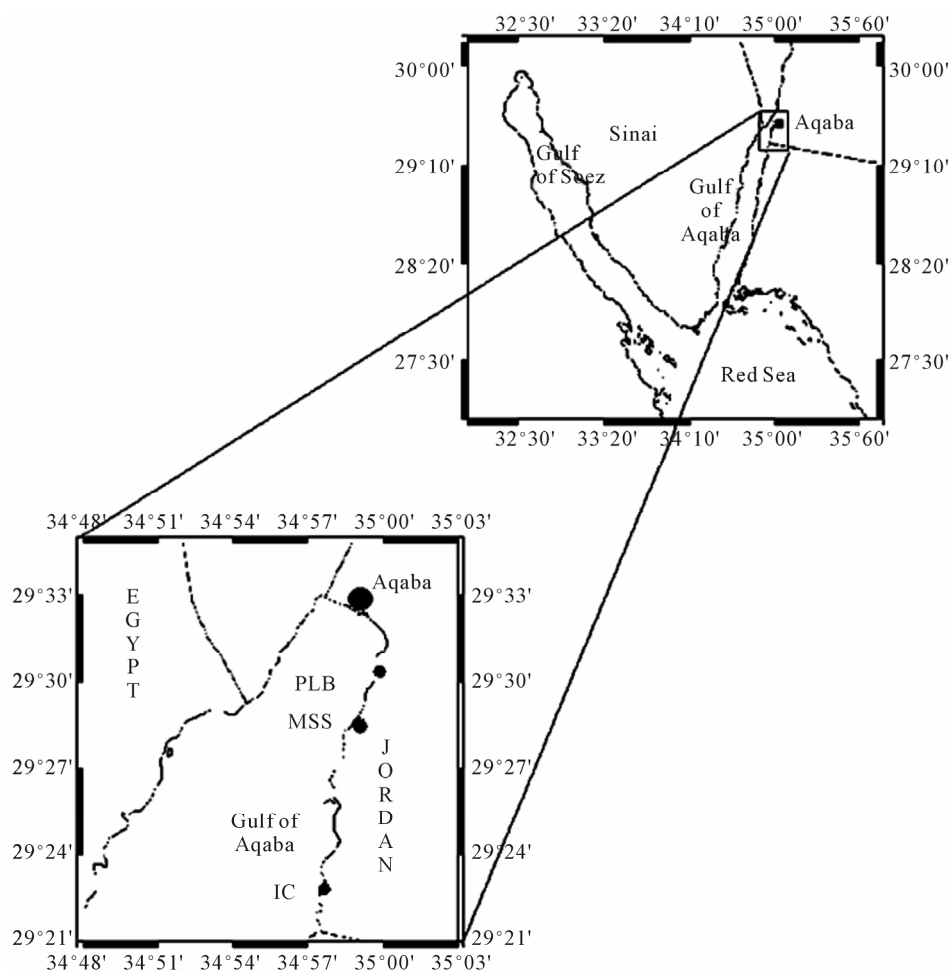
Fungi are found ubiquitously in the environment. They execute a wide range of important ecological functions especially ones associated with decomposition of organic substrates in both terrestrial as well as marine environments, and occasionally in extreme conditions [1-5]. The magnitude of fungal biodiversity was estimated to be 1.5 million species [6,7]. However, some estimation exceeded this number to 5.1 M [8]. Among the estimated 1.5 million, only 5% - 10% has been formally described [3,8]. Fungi that live in the sea are defined as obligate

marine or facultative. Obligate marine fungi are those that grow and sporulate exclusively in a marine or estuarine habitat and are permanently or intermittently submerged in water. Whereas, facultative marine fungi are those that normally occupy freshwater habitats or terrestrial surroundings but are able to grow (and possibly to sporulate) in the marine environment [9,10]. Currently there are 444 known recognized species of obligate and facultative marine fungi. *Ascomycota* represented by 360 species whereas *Basidiomycota* are represented by 10 species and 74 species of mitosporic fungi [11]. Facultative marine fungi can be transferred to seawater by wind, rain or runoff soil. Some facultative marine fungi were able to evolve adaptation to marine environment and eventually became obligate marine [12].

Many reasons have posed the need to examine the extent of marine biodiversity of fungi. For example, the emergence marine life diseases to corals, sponges, sea fan corals and fish [3,13-15]. Some marine fungi are reported as sources of enzymes used in bioremediation [16-18]. New biosynthetic products have been revealed in marine-derived fungi [19-21]. Moreover, marine fungi can be exploited for monitoring marine environment pollution [22].

Literature search revealed no studies examining fungal biodiversity in Gulf of Aqaba, a semi-enclosed water body located in the most southern part of Jordan and at the northern end of the Red Sea. Only one study examined manglicolous fungal diversity in of the Red Sea in the upper Egypt [23].

The Gulf of Aqaba is a sub-tropical arid area between longitude 34°25' to 35°00'E and latitude 28°00' to 29°33'N (Figure 1). The Gulf is unique for its great depth (about 1830 m) in proportion to its width (maximum 25 km); the mean depth of the Gulf is about 800 m. Most of the rainfall in the region of the Gulf occurs during the period November-May. The average rainfall in Aqaba town is about 35 mm/yr. Daily temperature ranges between 14°C in January to 45°C in summer and evaporation rates of



**Figure 1.** Location of the sampling sites along the coast of the Gulf of Aqaba.

seawater between 200 and 365 cm/yr [24]. The aim of this study is to investigate the existence of fungal life in seawater and sediments of the Gulf of Aqaba and determine their phylotypes. Using molecular identification approach, we report the isolation and identification of 5 *Ascomycete* sp. Partial sequences of the 18S small subunit (ssu) rRNA gene were used as queries in BLASTN search that revealed 98% - 100% identity. Phylogenetic analyses revealed close relationship among the isolated fungi and their potential affiliation with *Ascomycetes*. We propose further studies examining fungal biodiversity in this unique marine environment.

## 2. MATERIALS AND METHODS

### 2.1. Sampling Sites

Water samples were collected using 100 ml screw-cap sterilized bottle, from three selected locations, namely; Marine Science Station (MSS); Phosphate Loading Berth (PLB) and Industrial Complex area (IC) by snorkeling, or by SCUBA diving at a depth of 5 m, during Fall 2010.

The sampling sites are shown in **Figure 1**. Meanwhile sediment samples were collected from the same location using similar 100 ml sterilized plastic bottle. The sampling was performed twice during 2 months period in the fall 2010. All samples were transferred to the laboratory by using icebox and cultured within 40 hours of arrival.

### 2.2. Fungal Isolation and Culture Conditions

Seawater samples were diluted 1/10 using 0.5 M sterile NaCl solution, whereas sediment samples were first washed with sterile NaCl solution to prepare wash off solution. From both preparations, 100  $\mu$ l of either diluted sample or wash off were taken to spread on 2.5% (w/v) Sabouraud Dextrose agar plates containing 50  $\mu$ g/ $\mu$ l ampicillin. To rule out any possible contamination during manipulation, negative control plates received only sterile NaCl washing solution and were exposed to circulating air inside the laminar flow during culturing. All plates were incubated for 3 days at 30°C. After incubation, cultured plates were screened to select isolated fungal colonies. Each selected colony was sub-cultured

several times to ensure obtaining pure culture. Colony selection was primarily based on morphological differences between colonies. Although some colonies exhibited only slight differences, yet they were selected for further analysis. Nine colonies were selected for further identification analysis. Spores were harvested using sterile cotton swab pre-wetted with salt-tween [0.1% NaCl plus 0.1% tween-80 (vol/vol)] then released in salt-tween solution. After that, spores were counted using hemocytometer. Approximately 6 million spores from each isolate/colony were inoculated into Erlenmeyer flasks containing 25 mL of Sabouraud Dextrose Broth 2.5% (w/v) 50 µg/µl ampicillin. The flasks were incubated for 48 h at 30°C and 150 rpm shaking. All inoculations and fungal handling were done under ClassII BIOAIR Laminar flow (Euroclone division, Italy) to avoid any possible air contamination.

### 2.3. Genomic DNA Extraction

Fungal mycelia of each isolate were collected by centrifugation at 10,000 rpm using regular bench-top centrifuge. Prior to that, microscopic examination was done to test the purity of the culture. Genomic DNA was extracted from 2 - 3 mg of mycelia using recombinant lyticase (Sigma, USA) and according to the protocol in wizard genomic DNA purification kit (Promega, USA).

### 2.4. Primers and 18S rRNA Gene PCR Amplification and Sequencing

Forward Primer 5'-ATT GGA GGG CAA GTC TGG TG-3' and reverse primer 5'-CCG ATC CCT AGT CGG CAT AG-3' bind to conserved regions of the fungal 18S ssu rRNA gene and enclosing containing the variable regions V7 and V9 as described before [25]. PCR amplification was carried out using ReadyMix™ Taq PCR Reaction Mix (Sigma, USA) which contains all required reagents except primers. PCR amplification was carried out in reaction volume of 50 µl of which 25 µl of ready mix Taq PCR which contained 1.5 units Taq DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.001% gelatin and 0.2 mM dNTP, in addition to 2 µl of 10 µM of each primers and 5 µl (0.25 µg/µL) DNA templates were added to each reaction mixture. Blank that contained only water instead of DNA, was used in each PCR run to check for DNA contamination.

DNA amplification was carried out by thermal cycler (Biorad, USA). The samples were denatured at 94°C for 2 minutes, followed by 30 cycles of repeated denaturation (30 s at 94°C), annealing (45 s at 55°C), enzymatic chain extension (60 s at 72°C) and a final extension at 72°C for 10 minute. Following amplification, PCR products were analyzed by 1% agarose gel electrophoresis in 1X TBE buffer (9 mM Tris-borate, 0.2 mM EDTA)

and analyzed after staining with ethidium bromide.

### 2.5. Sequence Analysis

PCR products were sequenced at the Macrogen Company sequencing facility using Applied Biosystems 3730 mXL automated DNA sequencer (Macrogen, Korea). For identification, sequences were submitted to BLA-STN (National Center for Biotechnology Information; <http://www.ncbi.nlm.nih.gov>). The 18S rRNA gene sequences from each species and the matched sequences from GenBank were aligned using ClustalW2 (EMBL-European Bioinformatics Institute-EBI; <http://www.ebiac.uk>). Phylogenetic analyses were performed using Phylogeny.fr [25]. Phylogenetic tree was reconstructed using the maximum likelihood method implemented in the PhyML program (v3.0 alRT) and graphed using TreeDyn (V198.3) [26].

## 3. RESULTS

### 3.1. Phenotypic Characterization

Fungi were cultured from form the sediment and seawater of the Gulf of Aqaba, Red seas.

All isolates grew equally well on Sabouraud Dextrose agar plates at 30°C. Most colonies grew up to 3 - 4 cm in diameter after 48 h incubation at 30°C. No colonies grew on the negative control plates. According to their growth characteristics and colony morphology, 9 well-isolated colonies were selected for further characterization and identification.

### 3.2. PCR Amplification of the 18S ssu rRNA

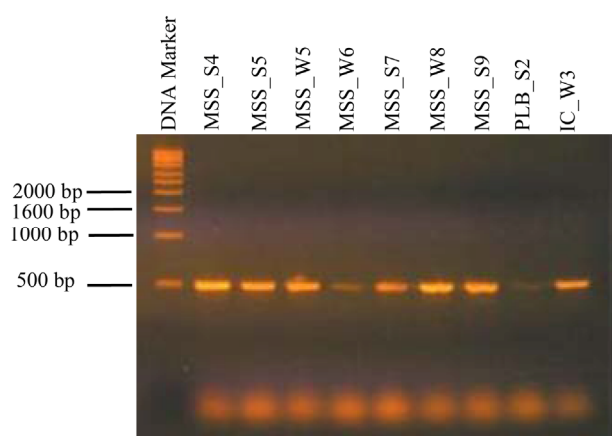
The primers target a consensus sequence of the 18S ssu rRNA gene containing the variable regions V7 and V9. Amplification with the primers described above is expected to yield a 482- to 503-bp fragment, depending on the fungus tested. **Figure 2** shows the amplicons resulted from PCR amplification. The figure does not reveal detectable size variation among amplicons.

### 3.3. 18S rRNA Gene Sequence Analysis

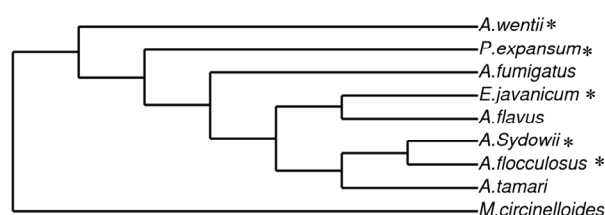
**Table 1** summarizes the results of the 18S ssu rRNA gene sequence analysis for these fungal isolates. The amplified fungal sequences were used as BLASTN queries against the NCBI database. The sequences of the PCR amplicons were found to be 98% - 100% similar to the sequences of 18S rRNA regions of the respective genera and species of closely related fungi. For example, the fungal sequence amplified from Marine Science Station sediment isolate no. 5 (MSS\_S5) showed 100% identity with an *Aspergillus sydowi* (Acc. No. JN938975) which was previously sequenced from *Aspergillus sydowii* strain DAOM 213727 18S ssu ribosomal RNA

**Table 1.** *Ascomycete* isolates identified in this study with substratum, site of isolation, GenBank BLASTN match Acc. Nos. of 18S rRNA sequence and percentage identity.

Isolate No.	Substratum	Site of isolation	Identification of closest relative	BLAST match Accession No.	Identity (%)
MSS_S4	Sediment	Marine Science Station	<i>Eupenicillium javanicum</i>	JN546126.1	100
MSS_S5	Sediment	Marine Science Station	<i>Aspergillus sydowi</i>	JN938975.1	100
MSS_W5	Seawater	Marine Science Station	<i>Eupenicillium javanicum</i>	JN546126.1	100
MSS_W6	Seawater	Marine Science Station	<i>Aspergillus flocculosus</i>	JN604547.1	100
MSS_S7	Sediment	Marine Science Station	<i>Eupenicillium javanicum</i>	JN546126.1	100
MSS_W8	Seawater	Marine Science Station	<i>Aspergillus wentii</i>	JN938967.1	98
MSS_S9	Sediment	Marine Science Station	<i>Penicillium expansum</i>	GU561988.1	99
PLB_S2	Sediment	Phosphate Loading Berth	<i>Penicillium expansum</i>	GU561988.1	99
IC_W3	Seawater	Industrial complex area	<i>Eupenicillium javanicum</i>	JN546126.1	100

**Figure 2.** Amplification products of 18S ssu rRNA gene of fungal isolates. DNA marker (number on the left are in kilobase); MSS\_S4, *Eupenicillium javanicum*; MSS\_S5, *Aspergillus sydowi*; MSS\_W5, *Eupenicillium javanicum*; MSS\_W6, *Aspergillus flocculosus*; MSS\_S7, *Eupenicillium javanicum*; MSS\_W8, *Aspergillus wentii*; MSS\_S9, *Penicillium expansum*; PLB\_S2, *Penicillium expansum*; IC\_W3, *Eupenicillium javanicum*. Fungal identification was based on closest relative resulted from BLASTN match.

gene partial sequence [27]. Similar patterns were observed amongst isolates of sediment MSS\_4 and seawater MSS\_W6 that also showed 100% identities to *Eupenicillium javanicum* and *Aspergillus flocculosus* respectively. Additionally, within our dataset, isolates from Marine Science Station sediment (MSS\_S9) showed 99% *Penicillium expansum* whereas seawater isolate MSS\_W8 exhibited 98% identity with *Aspergillus wentii*. Therefore, 5 different species belonging to 3 different genera have been isolated, all of which belong to phylum *Ascomycota*. No other fungal phyla have been isolated from these samples. Based on 18S ssu rRNA gene sequences, a phylogenetic tree analyses using Phylogeny.fr (**Figure 3**) represents phylogenetic positions of *Ascomycetes* iso-

**Figure 3.** Maximum likelihood phylogram inferred from partial 18S rDNA (450 base pairs) sequence data showing phylogenetic relationship of *Ascomycetes* isolated in this study (indicated in asterisks) to other *Aspergillus* sp. (*A. fumigatus*, *A. flavus* and *A. tamari*, Acc. No. HQ871898.1, JF824683.1 and AF516140.1 respectively) and *Zygomycete* (*Mucor circinelloides*, Acc. No. JN939019) as an outgroup. The phylogenetic tree was implemented in the PhyML program (v3.0 aLRT) and graphed using TreeDyn (V198.3).

lated in this study in relation to closely related *Aspergillus* sp. (*A. fumigatus*, *A. flavus* and *A. tamari*, Acc. No. HQ871898.1, JF824683.1 and AF516140.1 respectively) of which their 18S ssu rRNA sequences were obtained from GenBank. The selected *Aspergillus* sp. were among the list of hits obtained in BLASTN search. A *Zygomycete* (*Mucor circinelloides* Acc. No. JN939019) was used as an out-group. This results further supports observation that the isolated fungi are closely related and are most likely belong to the *Ascomycetes* group.

#### 4. DISCUSSION

The 18S small subunit rRNA gene is one of the most frequently used genes in phylogenetic studies and considered as a key target for environmental biodiversity screening [28-30]. In this study, we isolated fungi from three different locations that cover coastline of 26 kilometers long in the Gulf of Aqaba, Red sea. Identification of isolated fungi was achieved using PCR amplification of partial 18S ssu rRNA gene followed by DNA se-



quence analyses. Five different isolates belonging to 3 different genera were identified, namely; *Eupenicillium*, *Penicillium* and *Aspergillus* all of which belong to phylum *Ascomycota*. It has been well documented that *Ascomycetes* include a group of obligate marine fungi that tremendously contribute to marine ecosystem and represents variety of saprobes, pathogens and symbiont of coastal and deep-sea habitats [9,22,31,32]. Twenty six marine *Ascomycetes* have been previously isolated from mangrove wood and leaves in the intertidal zone of Red sea area at upper Egypt [22]. *Eurotiomycetes* of the phylum *Ascomycota* are the most frequently detected fungal taxa from deep-sea environments, followed by the classes *Saccharomycete*, *Dothideomycete* and *Sordariomycete*. The majority of species belonging to the *Eurotiomycete* are members of the *Aspergillus* and *Penicillium* genera, which are known to be globally distributed and ubiquitous in marine as well as terrestrial environments [5].

Although *Aspergillus* sp. is more commonly known as terrestrial fungi, many species of these mycelial fungi have been isolated from marine habitats. For example the ubiquitous existence of *A. sydowii*, *A. niger*, *A. wentii*, *A. flavus*, *A. insulicola* and *A. Awamori*. in marine environment have been established by both molecular and culture-dependent techniques [9,15,19,32-34]. In our study, two *Aspergillus* sp. isolated from sediment and seawater of the Gulf of Aqaba showed strong sequence homology to *A. sydowii* and *A. wentii* respectively. In addition, we isolated *Penicillium* sp. and *Eupenicillium* sp. from sediment samples. *Penicillium* has previously described as a marine-derived fungus [21]. However, to our knowledge, no reports have documented *Eupenicillium* sp. as a marine-derived fungus. It is anticipated that sequence data of 18S ssu RNA gene obtained from various sea environments will reveal more facultative marine fungi and perhaps novel fungal groups. Although members of *Basidiomycota* and lower fungi *Chytridiomycota* have been reported as inhabitants of marine fungi [5,9], we did not find any *Basidiomycete* or *Chytridiomycete* in our samples. Perhaps larger scale sampling will increase likelihood of isolating members of these groups in the Gulf of Aqaba.

Another aspect that highlights the importance of this study is based on the ability of fungi to overcome natural selection. Fungi to ensure well growth, sporulation and multiplication must barriers and acquire new capabilities that enable them adjust to new environmental conditions such as utilization of new carbon sources, adjustment to new osmotic—as well as hydrostatic—pressures and, in some instances, new temperature conditions [4,32]. Therefore, terrestrial fungi differ from its marine counterparts in; at least, metabolic activities and patterns of carbon source utilization. This suggests that, unlike terrestrial fungi, marine-derived fungi perhaps produce new sec-

ondary metabolites that can be exploited environmentally, biologically and perhaps pharmaceutically. For example, Sorbiterrin with a novel cholinesterase activity was isolated from marine-derived fungus *Penicillium terrestre* [20]. Cytotoxicity against selected cancer cells has been measured *in vitro* with compounds isolated from marine-derived fungus *Aspergillus insulicola* [19]. Marine derived fungi have shown their potential in polycyclic aromatic hydrocarbon bioremediation [16]. *Penicillium griseofulvum* as a eukaryotic organism has been exploited to screen heavy metal pollution in marine contaminated sediment [21]. Taken together, these studies indicate that filamentous fungi of marine environments might be considered as valuable sources of by-product compounds and enzymes that can be of industrial, pharmaceutical and environmental importance.

Our results report the presence of facultative marine *Ascomycetes* in the Gulf of Aqaba, Red sea and set the stage towards future investigations concerning marine fungal biodiversity in this area. We further propose exploration of marine-derived fungi as potential sources of valuable products.

## 5. ACKNOWLEDGEMENTS

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